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Association of polymorphisms and haplotypes in the human growth hormone 1 (GH1) gene with breast cancer

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Abstract

The growth hormone 1 (GH1)/insulin-like growth factor I (IGF-I) axis plays an important role in the development of breast cancer. By binding to its receptor, GH1 stimulates the production of IGF-I and its binding protein IGFBP3, resulting in the regulation of cell proliferation, differentiation and apoptosis. The GH1 gene expression is regulated by a highly polymorphic proximal promoter and a distal locus control region (LCR) 14.5 kb upstream of the gene. We investigated the effect of single nucleotide polymorphisms (SNPs) in the LCR and in the promoter region and an intron 4 SNP (IVS4 + 90 T/A) on breast cancer risk in a large cohort of Polish and German familial breast cancer cases and controls. SNPs in the LCR did not show an influence on breast cancer risk, either alone or in haplotypes. Three SNPs in the promoter region (G-340T, A-68G/C and A-63T/C) showed an increased and four SNPs (A-137G, G-119T, G-93delG and T-4G) a decreased allele frequency in the cases compared with the controls. Two of the SNPs (A-137G and G-93delG) lead to a decreased breast cancer risk among the minor allele carriers in the joint analysis of the two populations (odds ratio (OR) 0.62, 95% confidence interval (95% CI) 0.44–0.89, P=0.01 and OR 0.65, 95% CI 0.47–0.90, P=0.01, respectively). Haplotype analysis with these seven promoter SNPs revealed a protective association (OR 0.61, 95% CI 0.37–1.00, P=0.04) for the haplotype GAGdAAT, containing the G-93delG variant allele, which in the single analysis already showed a protective effect. The effect was marginally stronger in combination with the LCR GC haplotype (OR 0.49, 95% CI 0.23–1.01, P=0.04).

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Introduction

Growth hormone 1 (GH1) plays an important role in normal postnatal growth. It is also required for the normal development of the mammary gland (Kleinberg 1998). GH1 is synthesized in the pituitary and released into the circulation. It binds to the GH1 receptor (GHR) and stimulates the production of insulin-like growth factor I (IGF-I) and its binding protein IGFBP3, both known to act in the IGF-I pathway to regulate cell proliferation, differentiation and apoptosis (Laban *et al.* 2003). Acting through a feedback loop, circulating IGF-I can inhibit GH1 secretion (Le Roith *et al.* 2001). GH1 also has many IGF-I-independent effects on cellular growth, even though the main effects of GH1 on growth are IGF-I-dependent. Besides their endocrine effects, GH1 and IGF-I can act through autocrine and paracrine mechanisms in local tissues.

There is increasing evidence for the importance of the GH1/IGF-I axis in the development of breast cancer. Early findings by Emerman *et al.* (1985) showed that GH1 levels are elevated in breast cancer patients and, together with the more recent data on

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GH1 action, a pivotal role may be attributed to GH1 in the development of breast cancer. Increased IGF-I levels have been shown to be a risk factor for breast cancer (Renehan *et al.* 2004, Shi *et al.* 2004). Several components of this pathway show increased expression in breast cancer tissues (Laban *et al.* 2003). Transgenic mice overexpressing GH1 have been shown to develop mammary adenocarcinoma (Tornell *et al.* 1992). Pathological pituitary GH1 hypersecretion in humans is known as acromegaly. However, whether acromegaly predisposes to cancer is still controversial (Colao *et al.* 2004). *lit/lit* mice with a mutation in the GH-releasing hormone receptor have dramatically reduced GH1 and IGF-I levels, leading to reduced growth of mammary tumour transplants (Yang *et al.* 1996). There are also indications that autocrine GH1 has direct proliferative and antiapoptotic effects in human mammary carcinoma cell lines (Kaulsay *et al.* 2001). Finally, autocrine production of GH1 has been shown to lead to an invasive phenotype of mammary carcinoma cells (Mukhina *et al.* 2004).

The contribution of genetic factors in the regulation of GH1 secretion is about 27% during a 24-h period (Mendlewicz *et al.* 1999). The promoter of GH1 is highly polymorphic and a number of single nucleotide polymorphisms (SNPs) have been reported (Giordano *et al.* 1997, Wagner *et al.* 1997, Hasegawa *et al.* 2000, Le Marchand *et al.* 2002, Horan *et al.* 2003). Expression of GH1 has been shown to depend on the promoter haplotypes (Horan *et al.* 2003). So far, one study has investigated the effect of five SNPs in the promoter on breast cancer risk in a Chinese population, finding no effect (Ren *et al.* 2004). A further SNP in intron 4 (IVS4+90 T/A) has been shown to be in a 90% linkage disequilibrium (LD) with the promoter polymorphisms G-340T and T-119G and to be associated with decreased GH1 and IGF-I levels (Hasegawa *et al.* 2000). Recently, the intron 4 A allele has been associated with a lower risk of colorectal cancer (Le Marchand *et al.* 2002).

The GH1 gene is regulated by a locus control region (LCR) 14.5 kb upstream of the gene (Jones *et al.* 1995). Three SNPs have been reported in this region (Horan *et al.* 2003). Haplotypes of the LCR have been shown to regulate tissue-specific expression of the GH1 promoter in a promoter haplotype-dependent manner (Horan *et al.* 2003).

In the present study, we investigated the effect of the SNPs within the GH1 promoter, LCR and the intron 4 SNP (IVS4+90 T/A) on breast cancer risk primarily in a Polish familial breast cancer series. An independent German familial breast cancer series was used to confirm the results.

Material and methods

Subjects

The analyses were performed on genomic DNA with a maximum of 463 Polish familial breast cancer cases (mean age 46 years, range 22–81 years) and 470 regionally and ethnically matched female controls (mean age 40 years, range 16–76 years). The allele frequencies in our control population represented the allele frequencies in the general population. The inclusion criteria for the cases were (i) at least two first-degree relatives with breast and/or ovarian cancer regardless of age, (ii) breast cancer diagnosed below the age of 35 without family history, (iii) bilateral breast cancer regardless of the family history, (iv) breast and ovarian cancer diagnosed in one patient regardless of the family history and (v) breast cancer diagnosed below 50 years of age regardless of family history (Forsti *et al.* 2002, Jin *et al.* 2004). The subjects corresponding to criteria i–iv, 388 cases, were collected during the years 1997–2002 by the Chemotherapy Clinics and the Genetic Counselling Service (Gliwice, Poland) and the subjects corresponding to criterion v, 75 cases, were collected between December 2002 and March 2004 by the Surgery Clinics (Gliwice, Poland). No information about the number of cases belonging to each of categories i–iv was available. All cases were unrelated. They were tested for four founder mutations in BRCA1 and two in BRCA2 and were found to be negative. These mutations account for more than 90% of the BRCA1/2 mutations in the Polish population (Gorski *et al.* 2004).

An independent population consisting of 217 German familial breast cancer cases (mean age 42 years, range 17–68 years) and 243 regionally and ethnically matched female controls (mean age 37 years, range 4–79 years) was used to confirm the positive findings in the Polish population. This population was collected through the Institute of Human Genetics, University of Heidelberg (Heidelberg, Germany), from families with two or more cases of breast cancer including at least two cases with onset under the age of 50 years (A1; 61 cases), at least one male breast cancer (A2; five cases), one or more cases of breast and at least one ovarian cancer (B; 30 cases), two or more cases of breast cancer including one case diagnosed before the age of 50 (C; 96 cases), two or more cases of breast cancer diagnosed after the age of 50 years (D; five cases) and a single case of breast cancer with the age of diagnosis below 35 years (E; 16 cases). All cases were unrelated. The entire coding regions of the BRCA1 and BRCA2 genes were screened and cases carrying

deleterious BRCA1/2 mutations were excluded (Meindl 2002).

We used familial cases because it has been shown that selection of cases based on the family history of the same disease increases the power to detect low-penetrance variants (Antoniou & Easton 2003, Houlston & Peto 2003). About 90% of the patients and the controls approved participation to the study. The study was approved by the ethical committee of the University of Heidelberg.

Analysis of the promoter polymorphisms

The human GH locus contains five genes of high sequence homology (Chen *et al.* 1989). We investigated the SNPs in the promoter region of the GH1 gene by sequencing. First, we amplified a 951 bp fragment specific for GH1 in a 10 µl PCR using 5 ng genomic DNA, 1× PCR buffer (Invitrogen, Paisley, UK), 1.5 mM $MgCl_2$ (Invitrogen), 0.11 µM dNTP mixture (Invitrogen), 0.15 µM of each primer (forward, 5′-TG GTTTCAGGGCTATGGG-3′; reverse, 5′-GCTTA CATGGCGATACTCACA-3′; MWG Biotech AG, Ebersberg, Germany) and 0.3 U Platinum *Taq* polymerase (Invitrogen). The PCR programme was as follows: 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 6 min. PCR was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR product was cleaned up using 0.75 µl ExoSapIT (USB Amersham, Uppsala, Sweden) for 40 min at 37 °C and 15 min at 85 °C. The sequencing reaction was carried out as described by Wagner *et al.* (2004) using, in addition to the primers mentioned above, the following primers: Seq1, 5′-ACGGGCTTGTGCTAATGG-3′; Seq2, 5′-GTGTGGGGTTGGTTCTCTCTA-3′.

Analysis of the LCR polymorphisms

We first sequenced a 358 bp fragment within the LCR locus in a set of 23 breast cancer cases and confirmed the three SNPs reported earlier (Horan *et al.* 2003). The SNPs located at the nucleotide positions 1144 and 1194 (numbering according to Jin *et al.* (1999); GenBank accession no. AF010280) were linked in 22 out of 23 samples, whereas the SNP at position 990 was not linked to any of them. Therefore, we went on investigating the SNPs located at positions 990 and 1194. The SNP located at position 990 was investigated by restriction fragment length polymorphism (RFLP) analysis. PCR amplification was performed with 1× PCR buffer, 1.5 mM $MgCl_2$, 0.11 µM dNTPs, 0.15 µM of each primer (forward, 5′-TTCTGGGGTACAGG-TAGTTT-3′; reverse, 5′-GGAGTCTCATGGTTTA-GGAA-3′) and 0.3 U Platinum *Taq* polymerase. The reaction was performed at 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final extension of 6 min at 72 °C. The PCR product was digested with 5 U HhaII (MBI Fermentas, StLeon-Rot, Germany) at 37 °C overnight and the resulting fragments (GG, 288 + 70 bp; GA, 358 + 288 + 70 bp; AA, 358 bp) were visualized on a 2.5% agarose gel. About 10% of the RFLP results were confirmed by sequencing. For the SNP located at position 1194 allelic discrimination analysis was used (Assay-by-Design; Applied Biosystems). The assay was performed as described earlier (Wagner *et al.* 2004) and the assay information is available upon request from the corresponding author (K W).

RFLP analysis of the intron 4 polymorphism

For amplification of the intron 4 SNP (IVS4 + 90T/A; rs2665802; NCBI dbSNP), which is also described in the literature as T1663A or T1169A (Hasegawa *et al.* 2000, Le Marchand *et al.* 2002, Ren *et al.* 2004), we used a nested PCR and RFLP analysis described by Le Marchand *et al.* (2002) with minor modifications. First, a fragment of 541 bp specific for GH1 was amplified in a 10 µl reaction with 5 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP mixture, 0.1 µM of each primer (forward 1, 5′-TGACTTTGA-GAGCTGTGTTA-3′; reverse 1, 5′-AGAAGGACAC-CTAGTCAGACA-3′; Hasegawa *et al.* 2000) and 0.3 U Platinum *Taq* polymerase. The reaction was carried out at 94 °C for 2 min, followed by 22 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 6 min. 1 µl of this PCR product was used as a template for a 20 µl reaction using 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP mixture, 0.2 µM of each primer (forward 2, 5′-GAGA-AACACTGCTGCCCTCTTTTTAGACG-3′; reverse 2, 5′-AAGAGAAGGAGAGGCCAAGC-3′; Le Marchand *et al.* 2002) and 0.3 U Platinum *Taq* polymerase. The reaction was carried out at 94 °C for 2 min, followed by 22 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 6 min. 5 U of AatII (MBI Fermentas) was added to the PCR product. The resulting fragments (TT, 149 bp; TA, 149 + 179 bp; AA, 179 bp) were analyzed on ethidium bromide-stained 8% PAGE Mini-gels (Biorad Gel Casting System). The temperature and digestion time used were as recommended by the manufacturer. About 10% of the RFLP assay was randomly repeated and results were checked for

concordance. Additionally, we confirmed about 5% of the RFLP results by DNA sequencing.

Statistical analysis

The observed genotype frequencies in the breast cancer cases and controls were tested for Hardy–Weinberg equilibrium (HWE) and the difference between the observed and expected frequencies was tested for significance using the χ^2 test. Statistical significance for the differences in the genotype and haplotype frequencies between the breast cancer cases and controls was determined by the χ^2 test. The joint analysis was carried out using Mantel–Haenszel adjustment. Whenever the expected number of cases was less than five, Fisher's exact test was used. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for associations between genotypes and breast cancer. As menopausal status of the women has been shown to affect the levels of the IGF-I and IGFBP3 proteins (Renehan *et al.* 2004, Shi *et al.* 2004), we adjusted the results according to the age of diagnosis, less than and equal to or greater than 50 years. All calculations were carried out using the HWE test tool offered by the Institute of Human Genetics, TU Munich, Germany (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl.) and Epi Info 2000 software.

Haplotype analysis

LD between the SNPs was evaluated using the Haploview program (http://www.broad.mit.edu/mpg/haploview/documentation.php). Haplotypes were inferred using the SNPHAP program created by David Clayton (http://www.gene.cimr.cam.ac.uk/clayton). Haplotype effects were estimated by logistic regression analysis using the Statistical Analysis System software (version 9.3; SAS Institute, Cary, NY, USA).

Results

Polymorphisms in the GH1 promoter are associated with breast cancer risk

We screened the promoter region of the GH1 gene for polymorphisms in a small sample set of 23 breast cancer cases. We confirmed 16 out of the 22 SNPs reported so far by the NCBI database and by different laboratories (Giordano *et al.* 1997, Wagner *et al.* 1997, Hasegawa *et al.* 2000, Horan *et al.* 2003). We continued to investigate the promoter region in the Polish cohort. The allele frequencies of the SNPs among the cases and the controls are shown in Table 1. They are consistent with the allele frequencies reported in the Caucasian populations (Le Marchand *et al.* 2002, Horan *et al.* 2003, Ren *et al.* 2004). We restricted the further analyses to the SNPs with at least 4% minor allele frequency in the control population. We found three polymorphisms (SNPs 7, 14 and 15) to have a higher allele frequency in the cases than controls and four polymorphisms (SNPs 11, 12, 13 and 21) to show a decreased allele frequency in the cases compared with the controls.

We tested the association of the SNPs with breast cancer risk in the Polish population. All genotype distributions followed the HWE. Because GH1 is the main regulator of IGF-I, and IGF-I has been shown to have a different effect on breast cancer risk depending on the menopausal status of the women (Renehan *et al.* 2004, Shi *et al.* 2004), we divided the cases according to the age at diagnosis, below and equal to or greater than 50 years. Due to the missing age data, the total number of cases and controls may differ from the sum of the two age groups. SNPs 7 and 14 showed an increased risk for breast cancer (Table 2). For SNP 7 the increased risk was detected only in women diagnosed below age 50 years, whereas for SNP 14 both age groups were at equally high risk, which was significant in the whole population. For both SNPs the risk increased with an increasing number of variant alleles (Table 2). SNP 15 was not associated with the risk of breast cancer. For SNPs 11, 12, 13 and 21 there was a tendency for a decreased OR in the variant allele carriers (Table 2). However, statistically significant risks were observed only among carriers of the variant alleles of SNPs 13 and 21. We also investigated the SNP in intron 4 (IVS4+90 T/A) in our Polish sample set and found no difference in the allele or genotype distribution in the whole Polish population (Table 2). However, among women diagnosed for breast cancer below age 50 years an increased OR with an increasing number of the A alleles was observed, and in women homozygous for the A allele the OR of 1.55 (95% CI 0.95–2.52, $P = 0.08$) was of borderline significance.

We added the German cohort to our study to confirm the findings in the Polish cohort (Table 2). In this population, the controls for SNP 7 and the cases for SNP 14 deviated marginally from the HWE ($P = 0.03$ and 0.02, respectively.) All the other genotype distributions were in HWE. None of the SNPs 7, 14 and 15, which were more frequent in the Polish cases than controls, showed an effect on breast cancer risk in the German population. Of SNPs 11, 12, 13 and 21 which were less-frequent in the Polish cases than controls, SNP 11 showed a significant protective effect in the total German population. The effect was restricted to the cases diagnosed below age 50 years. For SNPs 12

Table 1 GH1 polymorphisms investigated in the present study

SNP	Position	NCBI SNP ID	Minor allele frequency cases*	Minor allele frequency controls*
LCR†				
1	G990A	Jin *et al.* 1999	7.4	9.3
2	A1144C‡	Jin *et al.* 1999		
3	C1194T‡	Jin *et al.* 1999	42.3	39.7
Promoter§				
1	G-538A	Wagner *et al.* 1997; Horan *et al.* 2003	4.6	3.3
2	G-435A	rs2005170	0.0	0.0
3	G-426T	Wagner *et al.* 1997; Horan *et al.* 2003	0.0	0.0
4	G-401DelG	Wagner *et al.* 1997; Horan *et al.* 2003	1.7	2.8
5	G-370T	rs1811081	24.3	24.3
6	G-363T	rs2011732	24.4	24.5
7¶	G-340T	rs2005171	47.1	41.8
8	CCAGA-334GAGAG	Wagner *et al.* 1997	0.0	0.0
9	C-298T	rs7219235	0.0	0.0
10	T-230C	rs2727338	1.9	2.3
11**	A-137G	rs11568828	6.6	9.1
12**	G-119T	rs2005172	31.4	36.5
13**	G-93delG	rs11568827	9.3	13.9
14¶	A-68G/C	rs6171	48.6	42.3
15¶	A-63T/C	rs695	12.8	10.7
16	G-60C	rs6175	1.2	0.2
17	A-47G/C	rs9282699	2.7	2.0
18	C-40T	Wagner *et al.* 1997	0.0	0.0
19	A-38C/G	rs6172	2.2	2.4
20	A-37C	Giordano *et al.* 1997	0.0	0.0
21**	T-4G	rs6173	2.4	4.5
22	A+7G	rs1805274	0.9	0.8
Intron 4	IVS+90 T/A	rs2665802	40.9	41.4

*allele frequencies in the Polish cohort.
†numbering according to AF010280 (Jin *et al.* 1999), SNPs at positions 990 and 1194 were analyzed further.
‡A1144C and C1194T were in nearly 100% LD.
§polymorphisms in the promoter that are highlighted were used for further analysis.
¶allele frequency is increased in cases compared to controls.
**allele frequency is decreased in cases compared to controls.

and 13, a similar decrease in the OR was seen as in the Polish cohort, although the effect was not significant. SNP 21 did not have any effect on breast cancer risk in the German population (Table 2).

The joint analysis with the Mantel–Haenszel adjustment confirmed the protective effect of the SNPs 11 and 13 with ORs of 0.62 (95% CI 0.44–0.89, $P = 0.01$) and 0.64 (95% CI 0.46–0.89, $P = 0.01$), respectively, for carriers of the variant alleles. Even though these protective effects were observed both in women diagnosed for breast cancer below and over 50 years, they were statistically significant only in the group of younger women. SNP 12 showed a decreased OR with an increasing number of variant alleles. Homozygotes for the variant allele had an OR of 0.66 (95% CI 0.43–1.01, $P = 0.05$) with a borderline significance in the whole population. None of the other SNPs were significantly associated with the risk of breast cancer.

Thus, the joint analysis of the Polish and German cohorts showed that three SNPs were associated with a decreased risk of breast cancer (SNPs 11, 12 and 13). None of the SNPs increased the risk for breast cancer.

LCR polymorphisms are not associated with breast cancer risk

We investigated two SNPs at positions 990 and 1194 in the LCR in the Polish cohort. No differences in the genotype distribution between the cases and the controls were observed, and nor did any haplotype

Table 2 Genotype distribution of the polymorphisms in the GH1 gene in the Polish and German cohorts

		Polish				German				Joint	
NCBI SNP	**Genotype**	cases	controls	OR (95% CI)	*P*-value	cases	controls	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
SNP 7											
<Age 50	GG	46 (0.21)	47 (0.30)	1		65 (0.40)	64 (0.38)	1		1	
	GT	118 (0.55)	82 (0.53)	1.47 (0.90–2.41)	0.13	70 (0.43)	70 (0.42)	0.99 (0.61–1.59)	0.95	1.19 (0.83–1.71)	0.35
	TT	51 (0.24)	27 (0.17)	**1.93 (1.04–3.58)**	***0.04***	26 (0.16)	34 (0.20)	0.75 (0.41–1.40)	0.37	1.21 (0.77–1.90)	0.45
	T%	51.2	43.6			37.8	41.1				
≥Age 50	GG	27 (0.38)	16 (0.40)	1		15 (0.34)	26 (0.45)	1		1	
	GT	37 (0.51)	16 (0.40)	1.37 (0.58–3.21)	0.47	18 (0.41)	24 (0.41)	1.30 (0.49–3.45)	0.56	1.34 (0.69–2.58)	0.44
	TT	8 (0.11)	8 (0.20)	0.59 (0.19–1.89)	0.37	11 (0.25)	8 (0.14)	2.38 (0.79–7.24)	0.12	1.08 (0.45–2.59)	0.99
	T%	36.8	40.0			45.5	37.9				
Total	GG	76 (0.26)	74 (0.32)	1		83 (0.38)	90 (0.39)	1		1	
	GT	156 (0.54)	122 (0.53)	1.25 (0.84–1.85)	0.28	96 (0.44)	96 (0.41)	1.08 (0.70–1.67)	0.70	1.16 (0.87–1.57)	0.33
	TT	59 (0.20)	36 (0.16)	1.60 (0.95–2.69)	0.08	38 (0.18)	45 (0.20)	0.92 (0.54–1.55)	0.74	1.21 (0.82–1.78)	0.36
SNP 11											
<Age 50	AA	188 (0.88)	117 (0.83)	1		134 (0.85)	110 (0.75)	1		1	
	AG+GG	26 (0.12)	24 (0.17)	0.67 (0.35–1.28)	0.20	23 (0.15)	37 (0.25)	**0.51 (0.27–0.94)**	**0.02**	**0.58 (0.38–0.90)**	**0.01**
	G%	6.5	8.9			7.6	13.3				
≥Age 50	AA	61 (0.86)	30 (0.75)	1		33 (0.77)	41 (0.77)	1		1	
	AG+GG	10 (0.14)	10 (0.25)	0.49 (0.17–1.45)	0.15	10 (0.23)	12 (0.23)	1.04 (0.36–2.98)	0.94	0.72 (0.34–1.52)	0.45
	G%	7.0	12.5			12.8	12.3				
Total	AA	253 (0.88)	196 (0.83)	1		180 (0.84)	154 (0.75)	1		1	
	AG+GG	36 (0.13)	41 (0.17)	0.68 (0.41–1.14)	0.12	34 (0.16)	51 (0.25)	**0.57 (0.34–0.95)**	**0.02**	**0.62 (0.44–0.89)**	**0.01**
SNP 12											
<Age 50	GG	108 (0.50)	68 (0.43)	1		79 (0.49)	76 (0.45)	1		1	
	GT	90 (0.42)	74 (0.46)	0.77 (0.50–1.18)	0.23	60 (0.37)	64 (0.38)	0.90 (0.56–1.45)	0.67	0.83 (0.59–1.15)	0.27
	TT	17 (0.08)	18 (0.11)	0.60 (0.29–1.23)	0.16	22 (0.14)	28 (0.17)	0.76 (0.40–1.44)	0.39	0.68 (0.41–1.13)	0.15
	T%	28.8	34.4			32	35.7				
≥Age 50	GG	24 (0.33)	16 (0.40)	1		21 (0.48)	23 (0.39)	1		1	
	GT	40 (0.56)	16 (0.40)	1.67 (0.71–3.93)	0.24	18 (0.41)	28 (0.47)	0.70 (0.31–1.63)	0.41	1.07 (0.59–2.04)	0.94
	TT	8 (0.11)	8 (0.20)	0.67 (0.21–2.14)	0.49	5 (0.11)	8 (0.14)	0.69 (0.19–2.42)	0.56	0.67 (0.26–1.73)	0.50
	T%	38.9	40.0			31.8	37.3				
Total	GG	134 (0.46)	96 (0.41)	1		104 (0.48)	102 (0.44)	1		1	
	GT	131 (0.45)	109 (0.46)	0.86 (0.60–1.24)	0.42	85 (0.39)	92 (0.40)	0.91 (0.61–1.35)	0.63	0.88 (0.87–1.12)	0.40
	TT	26 (0.09)	32 (0.14)	0.58 (0.33–1.04)	0.07	28 (0.13)	37 (0.16)	0.74 (0.42–1.30)	0.30	0.66 (0.43–1.01)	0.05
SNP 13											
<Age 50	GG	184 (0.86)	129 (0.77)	1		133 (0.84)	129 (0.77)	1		1	
	G/del+del/del	31 (0.14)	39 (0.23)	**0.56 (0.32–0.97)**	**0.03**	26 (0.17)	39 (0.23)	0.65 (0.36–1.16)	0.12	**0.60 (0.40–0.89)**	**0.01**
	del%	8.1	12.5			8.5	12.2				
≥Age 50	GG	55 (0.76)	26 (0.65)	1		36 (0.86)	45 (0.76)	1		1	
	G/del+del/del	17 (0.23)	14 (0.35)	0.57 (0.23–1.46)	0.26	6 (0.14)	14 (0.23)	0.54 (0.16–1.69)	0.24	0.56 (0.27–1.13)	0.12

Table 2 *continued*

		Polish				German				Joint	

Table 2 *continued*

NCBI SNP	Genotype	Polish				German				Joint	
		cases	controls	OR (95% CI)	*P*-value	cases	controls	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
	del%	12.5	21.2			7.1	13.6				
Total	GG	243 (0.83)	183 (0.75)	1		178 (0.84)	179 (0.77)	1		1	
	G/del + del/del	51 (0.17)	61 (0.25)	**0.63 (0.41–0.98)**	**0.03**	35 (0.16)	53 (0.23)	0.66 (0.40–1.10)	0.09	**0.64 (0.46–0.89)**	**0.01**
SNP 14											
< Age 50	AA	42 (0.20)	49 (0.29)	1		61 (0.38)	64 (0.38)	1		1	
	AG	123 (0.57)	89 (0.52)	1.61 (0.98–2.64)	0.06	63 (0.39)	69 (0.41)	0.96 (0.59–1.58)	0.86	1.24 (0.86–1.78)	0.26
	GG	50 (0.23)	33 (0.19)	1.77 (0.97–3.23)	0.06	36 (0.23)	35 (0.21)	1.08 (0.60–1.93)	0.80	1.37 (0.88–2.13)	0.17
	G%	51.9	45.3			42.2	41.4				
≥ Age 50	AA	22 (0.31)	19 (0.48)	1		16 (0.36)	22 (0.37)	1		1	
	AG	40 (0.56)	16 (0.40)	2.16 (0.93–5.02)	0.07	17 (0.39)	29 (0.49)	0.81 (0.34–1.94)	0.63	1.34 (0.70–2.60)	0.42
	GG	10 (0.14)	5 (0.13)	1.73 (0.50–5.95)	0.38	11 (0.25)	8 (0.14)	1.89 (0.62–5.77)	0.26	1.81 (0.73–4.53)	0.23
	G%	41.7	32.5			44.3	38.1				
Total	AA	67 (0.23)	79 (0.32)	1		79 (0.37)	87 (0.37)	1		1	
	AG	163 (0.56)	127 (0.51)	**1.51 (1.02–2.26)**	**0.04**	89 (0.41)	100 (0.43)	0.98 (0.63–1.52)	0.92	1.23 (0.91–1.66)	0.18
	GG	60 (0.21)	41 (0.17)	**1.73 (1.03–2.88)**	**0.04**	48 (0.22)	46 (0.20)	1.15 (0.83–1.38)	0.59	1.40 (0.96–2.05)	0.08
SNP 15											
< Age 50	AA	162 (0.76)	113 (0.74)	1		123 (0.77)	131 (0.78)	1		1	
	AT/AC + TT/CC	53 (0.24)	39 (0.26)	0.95 (0.58–1.58)	0.85	37 (0.23)	36 (0.22)	1.09 (0.63–1.90)	0.73	1.02 (0.70–1.47)	0.99
	T/C%	13.3	13.2			13.8	12.3				
≥ Age 50	AA	57 (0.81)	33 (0.83)	1		39 (0.89)	48 (0.81)	1		1	
	AT/AC + TT/CC	14 (0.19)	7 (0.17)	1.16 (0.39–3.56)	0.77	5 (0.12)	11 (0.18)	0.56 (0.15–1.94)	0.31	0.84 (0.38–1.85)	0.78
	T/C%	9.7	8.8			8.0	11.0				
Total	AA	223 (0.77)	195 (0.79)	1		168 (0.78)	181 (0.79)	1		1	
	AT/AC + TT/CC	67 (0.23)	52 (0.22)	1.13 (0.73–1.73)	0.57	47 (0.22)	49 (0.21)	1.03 (0.64–1.66)	0.87	1.08 (0.79–1.48)	0.66
SNP 21											
< Age 50	TT	207 (0.96)	161 (0.94)	1		139 (0.91)	132 (0.94)	1		1	
	TG + GG	8 (0.04)	10 (0.06)	0.62 (0.22–1.75)	0.33	14 (0.09)	9 (0.06)	1.48 (0.58–3.84)	0.30	1.00 (0.51–1.98)	0.87
	G%	2.1	2.9			4.9	3.2				
≥ Age 50	TT	68 (0.94)	36 (0.95)	1		42 (0.95)	46 (0.88)	1		1	
	TG + GG	4 (0.06)	2 (0.05)	1.06 (0.19–6.06)	0.95	2 (0.04)	6 (0.12)	0.37 (0.05–2.17)	0.21	0.59 (0.17–2.06)	0.54
	G%	2.7	2.6			3.4	6.7				
Total	TT	278 (0.96)	234 (0.95)	1		189 (0.92)	183 (0.92)	1		1	
	TG + GG	13 (0.04)	23 (0.05)	**0.48 (0.21–1.01)**	**0.04**	17 (0.08)	15 (0.08)	1.10 (0.50–2.40)	0.80	0.71 (0.42–1.20)	0.21
IVS4 + 90											
< Age 50	TT	56 (0.26)	132 (0.33)	1							
	TA	112 (0.52)	198 (0.50)	1.33 (0.90–1.97)	0.15						

Table 2 *continued*

NCBI SNP	Genotype	Polish				German			Joint	
		cases	controls	OR (95% CI)	*P*-value	cases	controls	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
	AA	46 (0.21)	70 (0.18)	1.55 (0.95–2.52)	0.08						
	A%	48.6	42.3								
≥ Age 50	TT	26 (0.36)	17 (0.43)	1							
	TA	39 (0.54)	16 (0.40)	1.59 (0.69–3.71)	0.28						
	AA	7 (0.10)	7 (0.18)	0.65 (0.19–2.20)	0.49						
	A%	36.8	41.4								
Total	TT	158 (0.34)	159 (0.34)	1							
	TA	231 (0.50)	233 (0.50)	0.99 (0.75–1.33)	0.99						
	AA	74 (0.16)	78 (0.17)	0.97 (0.66–1.42)	0.87						
	A%	40.9	41.4								

The frequencies of cases and controls are shown in parenthesis.
Results with a *P*-value < 0.05 are highlighted in bold.

show a significant association with breast cancer risk (Table 3). Adjustment for age did not change the results. The control population for the SNP at position 1194 deviated slightly from the HWE ($P = 0.02$). As the LCR SNPs did not show any effect on the risk of breast cancer, we did not study them in the German cohort.

Haplotype analysis

We performed a LD analysis of the polymorphisms in the GH1 promoter in the Polish cohort (Table 4) and confirmed the earlier report of a high LD between the SNPs (Hasegawa *et al.* 2000). Also, the LCR SNPs and the IVS4 + 90 T/A SNP showed a high LD with all the promoter SNPs. Polymorphisms with a minor allele frequency of ≤ 4% were excluded from the analysis.

In the Polish cohort we combined the seven promoter SNPs described in Table 2 (SNPs 7, 11, 12, 13, 14, 15 and 21) for a haplotype analysis to investigate whether any haplotype was linked to breast cancer (Table 5). Only six haplotypes (H1–H6) appeared with a frequency of ≥ 5% in the control population. They accounted for ~85% of all the haplotypes. Inclusion of the intron 4 SNP in the promoter haplotype did not change the haplotype distribution (data not shown). Since the LCR regulates GH1 expression we added the LCR haplotypes to the promoter haplotypes (Table 5). As the LCR and the promoter SNPs were in high LD, each promoter haplotype, with the exception of H4, appeared mainly with one LCR haplotype, and we only show the combined results (Table 5).

When haplotypes with frequencies among controls of ≥ 1% were analysed, the results from logistic regression analysis indicated a haplotype effect ($P = 0.07$). Each haplotype was compared against all the other haplotypes in Table 5. Although this is a conservative method, it was used because the 'wild-type' haplotype (GC-H3) was only the third commonest. The LCR haplotype GC was mainly linked with the promoter haplotype H1 and with a frequency of 30% it was the commonest haplotype. It contained SNP 12, which alone had a marginal protective effect (see Table 2). The LCR haplotype GT was most frequently combined with promoter haplotype H2 with a frequency of 19% in the control population. It contained SNPs 7 and 14, which alone increased the risk of breast cancer (see Table 2). Haplotypes GT-H2 and GC-H3 were more common in cases than controls, but the differences were not statistically significant. The promoter haplotype H4 (GAGdAAT) was found to be protective with an OR of 0.61 (95% CI 0.37–1.00,

Table 3 Association of the genotypes and haplotypes of the GH1-LCR with breast cancer risk in the Polish cohort

	Cases	Controls	OR (95% CI)	*P*-value
bp 990				
GG	288 (0.85)	226 (0.82)	1	
GA + AA	50 (0.15)	49 (0.18)	0.83 (0.53–1.32)	0.42
bp 1194				
CC	107 (0.31)	89 (0.33)	1	
CT	182 (0.53)	150 (0.55)	1.01 (0.71–1.44)	0.96
TT	54 (0.16)	33 (0.12)	1.36 (0.81–2.28)	0.24
Haplotypes				
GC	343 (0.51)	279 (0.51)	1.00 (0.82–1.22)	0.97
GT	284 (0.42)	219 (0.40)	1.05 (0.85–1.30)	0.64
AC	49 (0.07)	49 (0.09)	0.81 (0.53–1.25)	0.32
AT	0 (0.00)	1 (0.01)	0.27 (0.01–6.65)	0.27

$P = 0.04$). This haplotype contained SNP 13, which also in the single SNP analysis showed a protective effect. The LCR haplotype GC marginally decreased the breast cancer risk of the carriers of the promoter haplotype H4 (OR 0.49, 95% CI 0.23–1.01, $P = 0.04$). Haplotype combinations containing the promoter haplotypes 1, 5 and 6 were equally common among cases and controls. Adjustment for age did not change the results.

We performed a haplotype analysis also with the six promoter polymorphisms identified by Horan *et al.* (2003) as the major determinants of GH1 expression. These polymorphisms correspond to our SNPs 1, 7, 10, 12, 14 and 17. The four most common haplotypes represented about 80% of all haplotypes. The other haplotypes appeared with a frequency of ≤ 5%. None of the haplotypes was associated with the breast cancer risk in any population (data not shown).

Discussion

Most of the growth-promoting effects of GH1 are mediated by the IGF-I pathway (Le Roith *et al.* 2001, Laban *et al.* 2003). GH1 is also the main regulator of IGF-I. Thus, changes in the expression of GH1 may influence cell proliferation, differentiation and apoptosis. The GH1 gene is located on chromosome 17q22–q24 within a cluster of five highly homologous genes expressed in a tissue-specific fashion (Chen *et al.* 1989). An LCR region upstream of the GH gene cluster is required for the activation of the different genes in a tissue-specific manner (Ho *et al.* 2004). GH1 is produced in the anterior pituitary gland driven by the pituitary-specific transcription factor Pit-1/GHF-1. A 203 bp region within the LCR is required for the expression of the GH1 gene and Pit-1/GHF-1-like DNA elements have been identified in this region

Table 4 Linkage disequilibrium, |D'|, between the LCR SNPs, the GH1 promoter SNPs and the IVS4 SNP in the Polish cohort. SNPs with a minor allele frequency of ≤ 0.04 were excluded

	SNP											
SNP	990*	1194*	5	6	7	11	12	13	14	15	21	IVS4
990*	–											
1194*	1.00	–										
5	0.84	0.82	–									
6	0.95	0.91	0.99	–								
7	0.95	0.93	0.85	0.85	–							
11	1.00	0.89	0.84	0.68	0.84	–						
12	0.70	0.86	0.60	0.61	0.78	0.78	–					
13	0.81	0.82	0.62	0.69	0.84	1.00	0.67	–				
14	0.75	0.77	0.37	0.66	0.75	0.70	0.82	0.71	–			
15	1.00	0.73	0.16	0.63	0.79	0.25	0.93	0.81	0.90	–		
21	0.70	0.63	0.62	0.63	0.69	1.00	1.00	0.68	0.55	1.00	–	
IVS4	0.88	0.71	0.91	0.85	0.71	0.56	0.55	0.57	0.60	0.57	0.60	–

*SNPs are located in the LCR.

Table 5 Combination of GH1 promoter haplotypes and LCR haplotypes and their association with breast cancer in the Polish cohort. GH1-promoter haplotypes are cited in the order of bases: SNP 7, 11, 12, 13, 14, 15, 21. GH1-LCR haplotypes are cited in the order of 990 bp and 1194 bp. Variant alleles are underlined

		GH-LCR								
		GC			GT			AC		
	GH promoter	cases	controls	OR (95% CI)†	cases	controls	OR (95% CI)†	cases	controls	OR (95% CI)†
H1	GATGAAT	161 (0.28)	145 (0.30)	0.95 (0.73–1.24)	2 (0.01)	5 (0.01)	0.34 (0.05–1.99)	0 (0.00)	0 (0.00)	
H2	TAGGGAT	7 (0.01)	3 (0.01)	2.00 (0.47–9.82)	134 (0.24)	91 (0.19)	1.27 (0.93–1.71)	0 (0.00)	0 (0.00)	
H3	GAGGAAT	58 (0.10)	34 (0.07)	1.47 (0.92–2.33)	1 (0.01)	1 (0.01)	0.86 (0.14–5.33)	1 (0.01)	6 (0.01)	0.14 (0.01–1.19)
H4	GAGdAAT*	13 (0.02)	23 (0.05)	**0.49 (0.23–1.01)**	0 (0.00)	0 (0.00)		18 (0.03)	20 (0.04)	0.77 (0.39–1.55)
H5	TAGGGTT	1 (0.01)	1 (0.01)	0.86 (0.02–31.44)	55 (0.10)	43 (0.09)	1.10 (0.71–1.70)	0 (0.00)	0 (0.00)	
H6	TGGGGAT	0 (0.00)	4 (0.01)	0.10 (0.01–1.78)	31 (0.05)	27 (0.06)	0.99 (0.56–1.73)	0 (0.00)	0 (0.00)	

*The deletion polymorphism SNP 13 is indicated by "d".
†ORs were calculated by comparing the given haplotypes against all other haplotypes.
The frequencies of cases and controls are shown in parenthesis.
Results with a *P*-value <0.05 is highlighted in bold.

(Jin Y. *et al.* 1999). However, GH1 is also produced locally in mammary epithelial cells, where it can act in an autocrine/paracrine manner (Raccurt *et al.* 2002, Laban *et al.* 2003, Mol *et al.* 1995). The GH1 mRNA expressed in the mammary gland is identical to the pituitary mRNA (Kaulsay *et al.* 2000). However, it is still unclear if the LCR is involved in mammary tissue-specific expression and other yet unidentified regulatory regions may exist.

The regulation of the GH1 gene transcription is also exerted by tissue-specific transcription factors that bind specific *cis*-elements located in the immediate 5′-flanking promoter region (Giordano *et al.* 1997, Wagner *et al.* 1997, Lantinga-van Leeuwen *et al.* 2002, Horan *et al.* 2003). In a recent study, Lantinga-van Leeuwen *et al.* (2002) have identified a putative progesterone-response element sequence and suggested a local *trans*-activation of the GH1 gene in mammary gland by ligand-activated progesterone receptors. The promoter of the GH1 gene is highly polymorphic, with 22 SNPs within a 550 bp stretch (Giordano *et al.* 1997, Wagner *et al.* 1997, Hasegawa *et al.* 2000, Horan *et al.* 2003). Interestingly, most of the polymorphisms in the proximal promoter are at positions that are strictly conserved in different species (Krawczak *et al.* 1999). However, at each polymorphic position, the human GH1 gene sequence differs from at least one of the other four genes within the GH gene cluster, and the variant allele is identical with at least one of the other genes (Giordano *et al.* 1997). This would suggest functional importance of this region. Investigations by electrophoretic mobility shift assays have revealed *cis*-acting regulatory sequences in the promoter region (Lantinga-van Leeuwen *et al.* 2002, Horan *et al.* 2003). Some of the SNPs studied here (SNPs 11, 12, 13, 15 and 19) are located within these sequences and have shown allele-specific protein binding (Horan *et al.* 2003). Different haplotypes in this region have been shown to lead to differences in GH1 expression. Horan *et al.* (2003) have identified six polymorphisms (corresponding to our SNPs 1, 7, 10, 12, 14 and 17) as major determinants of GH1 expression. When we performed a haplotype analysis with these polymorphisms we could not detect any effect on breast cancer risk.

Here, we investigated 22 SNPs in the proximal GH1 promoter and three SNPs in the LCR. Four SNPs in the GH1 promoter showed a decreased allele frequency in the cases compared with the controls but only two SNPs showed a significantly decreased breast cancer risk (SNPs 11 and 13). SNP 13 was also included in the only significantly protective haplotype, H4. The

protective effect of the haplotype H4 became even stronger in combination with the LCR GC haplotype. Even though multiple comparisons were done, the consistency of the effect of SNPs 11 and 13 in two independent populations as well as in the haplotype analyses made our findings more reliable. SNP 11 is located within the proximal Pit-1-binding site and SNP 13 5′ of the TATA box within a vitamin D-response element. Pit-1 is necessary for the pituitary expression of the GH1 gene (Lantinga-van Leeuwen *et al.* 2002) and it has been shown to be expressed in MCF-7 breast cancer cells (Gil-Puig *et al.* 2002). However, it is unlikely that Pit-1 plays a role in mammary expression of the GH1 gene (Lantinga-van Leeuwen *et al.* 2002). Three promoter SNPs showed an increased frequency in the cases compared with the controls but none of them alone, in a promoter haplotype alone or in combination with the LCR haplotypes, was associated with breast cancer risk.

Additionally, we investigated a T→A polymorphism in intron 4 (IVS+90 T/A) that has been associated with lower plasma levels of GH1 and IGF-I (Hasegawa *et al.* 2000) and decreased risk for colorectal cancer (Le Marchand *et al.* 2002). The effect of this SNP may be explained by its close linkage with the promoter SNPs, which has been shown by us in this study and earlier by others (Hasegawa *et al.* 2000). In our study, we observed a trend for an increased OR in women diagnosed below the age of 50 while in the total population no effect was found. Haplotype analysis together with the promoter SNPs did not show an effect.

To our knowledge, the only study on polymorphisms in the GH1 promoter region and cancer risk has been conducted in a Chinese population (Ren *et al.* 2004). This study did not find any effect of the SNPs 11, 12, 14 and 22, or the intron 4 SNP, or any haplotype on breast cancer risk. Ethnic differences in the effect of the intron 4 SNP on cancer risk have been discussed (Le Marchand *et al.* 2002, Ren *et al.* 2004), and nutritional factors have been suggested as an explanation.

In conclusion, the complexity of the GH1 gene regulation is reflected in our association study related to the polymorphisms within the GH1 gene region and the risk of breast cancer. Two of the SNPs (A-137G and G-93delG) lead to a decreased risk of breast cancer. The G-93delG variant allele was also involved in the only haplotype protecting against breast cancer. More data are needed on the tissue-specific regulation of the GH1 expression as well as on the influence of the polymorphisms on the expression and further on the risk of breast cancer.

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